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Chinese Medical Journal, 109(5): 372–375, 1996.

PROMOTION OF DIFFERENTIATION AND PROLIFERATION OF PERIPHERAL BLOOD CD34$^+$ CELLS IN VITRO BY G–CSF

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Objective. To observe the effect of granulocyte–colony stimulating factor (G–CSF) on differentiation and proliferation of CD34$^+$ cells from peripheral blood in presence of recombinant hematopoietic growth factor (HGF).

Methods. Peripheral blood mononuclear cells mobilized by G–CSF were obtained from patients suffering from carcinoma, preparing for autologous bone marrow transplantation. CD34$^+$ cells were isolated by derivatized polystyrene tissue culture flask which had covalently immobilized soybean agglutinin lectin and was coated with anti–CD34 antibody; and this kind of cells were incubated in liquid culture medium for up to 28 days under the stimulation of combination of growth factors, i.e., stem cell factor (SCF) and interleukin–3 (IL–3) with or without G–CSF. The changes of nucleated cells, colony forming unit–granulocyte and monocyte (CFU–GM), burst forming unit–erythrocyte (BFU–E), colony forming unit–megakaryocyte (CFU–MK), and myeloid–associated markers were evaluated.

Results. An increase of nucleated cells (mean 640–fold increase) occurred during culture. CFU–GM production is parallel to the nucleated cell production until the 11th day (mean 82–fold increase) in combination of 3 HGF, i.e., G–CSF, IL–3 and SCF. A large number of cells expressing late myeloid markers appeared on the 11th day in suspension culture of CD34$^+$ cells.

Conclusion. G–CSF was found to synergize with IL–3 and SCF in inducing rapid proliferation of purified CD34$^+$ cells and differentiation to multiple myeloid lineages. The stroma–free, cytokine–driven culture system could achieve a degree of amplification of colony forming cells, suggesting the feasibility of culture of hematopoietic progenitor cells in vitro as an adjunct to hematopoietic stem cell transplantation.

(Chin Med J 1996; 109(5): 372–375)

Granulocyte–colony stimulating factor (G–CSF) is a hematopoietic cytokine which mainly regulates granulocyte lineage hematopoiesis. It has unique and interesting characteristics among the family of hematopoietic growth factors. G–CSF interacts with other hematopoietic growth factors in stimulating proliferation of progenitor cells.[1] Our results indicated that G–CSF promoted differentiation and proliferation of peripheral blood CD34$^+$ cells in vitro.

MATERIAL AND METHODS

Peripheral blood mononuclear cells (PBMNC) for study. PBMNC were taken from patients with advanced breast carcinoma receiving chemotherapy as the treatment for their malignancy and also recombinant human G–CSF (Kirin Brewery Co., Ltd, Tokyo, Japan) 10 μg / kg daily for 7 successive days by subcutaneous injection during hematopoietic recovery after the period of myelosuppression. PBMNC, which were cryopreserved for 13–21 days, were obtained from aphereses.

Processing of cells. Cryopreserved PBMNC were thawed at 37℃ and slowly diluted in a 10× volume of precolded Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) with 20% fatal calf serum (FCS), centrifuged at 400 × g for 3 minutes, and half of the volume of the supernatant was then discarded, a 5× volume of precolded IMDM with 20% FCS was added slowly again, washed twice with this solution, and resuspended at 1×10^7 cells / ml.

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Isolation of $CD34^+$ cell. The low density cell fraction was separated by Ficoll gradient centrifugation (400 × g for 20 minutes). Adherent cells and T–lymphocytes were simultaneously depleted by adherence to the derivatized polystyrene tissue culture flasks (Applied Immune Science, Menlo Park, CA) which had covalently immobilized soybean agglutinin lectin (SBA). SBA–cells were incubated for 1 hour at room temperature in flasks coated with anti–CD34 antibody. The cells which were not adhered were removed with a pipette, and the adhered cells ($CD34^+$) were recovered by washing the flask with DPBS–CMF (Dulbecco's phosphate buffered saline Ca^{++} / Mg^{++} free, GIBCO, Grand Island, NY) containing 10% FCS. To dislodge the adherent cells, the device was hit sharply on its side one to three times. The flasks were rinsed with DPBS–CMF containing 10% FCS, and the pooled adherent cells were concentrated and used as desired.

Hematopoietic growth factors. The purified recombinant human hematopoietic growth factors included stem cell factor (SCF), granulocyte–colony stimulating factor (G–CSF), granulocyte monocyte–colony stimulating factor (GM–CSF), erythropoietin (Epo) and interleukin–3(IL–3). The concentrations were 100 μg / L for G–CSF, GM–CSF and SCF, 10 U / L for IL–3, and 2 U / ml for Epo (supplied by Amgen, Thousand Oaks, CA).

Liquid culture. $CD34^+$ cells were incubated in liquid culture medium for over 28 days. The culture medium consisted of IMDM supplemented with 5% FCS, 10% horse serum, and antibiotics (100 U penicillin, 100 μg streptomycin). The culture was stimulated with a combination of growth factors, i.e., SCF and IL–3 with or without G–CSF. The growth factors were added at the outset of the culture.

Clonogenic assay. After incubation for 5, 11, 15, 20 and 28 days at 37℃ in 5% CO_2, the contents of each well were resuspended and washed in IMDM to remove residual HGF. CFU–GM, BFU–E, CFU–MK assays were then made. Briefly, triplicate 1 ml culture was established in 35–mm plate in 1% methyl cellulose in IMDM supplemented with 20% FCS, 10% bovine serum albumin (BSA) and 2–mercaptoethanol (2–ME) (5×10^{-6}M, final concentration). Various combinations of growth factors were added to the dish at appropriate concentrations to stimulate colony growth, including Epo, IL–3 and SCF for BFU–E and CFU–MK growth, and GM–CSF, IL–3 and SCF for CFU–GM growth. The cells from liquid culture taken at different time points were placed at 6×10^3 cells / plate. After 14 days of incubation at 37℃ in 5% CO_2 and 100% humidity, CFU–GM, BFU–E and CFU–MK were identified and scored.

Immunophenotyping. A two–color immunolabeling procedure using directly conjugated monoclonal antibodies (McAbs) was performed to study the surface markers of the cells. The cells which were stained with the appropriate directly conjugated isotype antibodies were used as control. At least 10 000 events were collected in list mode on a FACScan.

RESULTS

Purity of isolated $CD34^+$ cells. Isolated $CD34^+$ cells were more than 97% pure as judged by flow cytometry.

Proliferative responses to HGF. Growth ability of isolated $CD34^+$ cells in the presence of HGF is shown in Fig.1A. The proliferation was markedly enhanced in culture stimulated with IL–3, SCF and G–CSF, reaching about 640–fold multiplication after 11–day culture. Cells produced in this system predominantly belonged to granulocyte

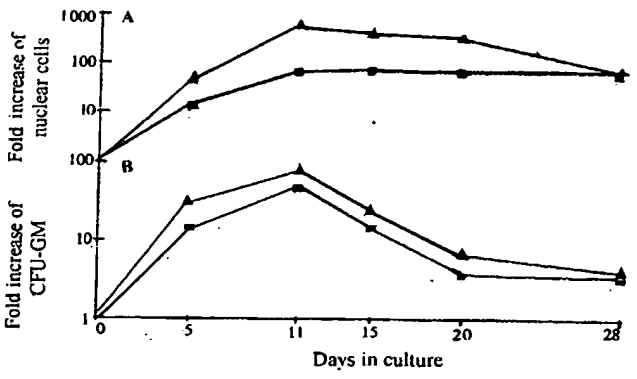

Fig. 1. A: the fold increase of nuclear cells in liquid culture of isolated $CD34^+$ cells. B: the fold increase of CFU–GM in the same sample. The results represent the mean of four separate experiments performed in triplicate (▲)IL–3+SCF+G–CSF, (■)IL–3+SCF.

Chinese Medical Journal, 109(5): 372–375, 1996.

lineage that was morphologically normal and acquired myeloid antigen, while IL–3 and SCF group (without G–CSF) induced a 78–fold multiplication during the same period ($P<0.001$).

Changes of CFU–GM production. The results are shown in Fig. 1B. The number of CFU–GM generated after 11 days for suspension culture, expressed an 82.2 (mean) fold increase over the stating number originated from 6×10^3 $CD34^+$ cells. In the group without G–CSF, CFU–GM had 50–fold increase ($P<0.01$).

Expression of myeloid–associated markers on the culture cells. Fig. 2 shows individual results of relative number of cells according to expression of 3 myeloid associated cell surface markers. G–CSF promoted more cells to express late myeloid markers (CD13, CD14) and erythroid markers (glycophorin A).

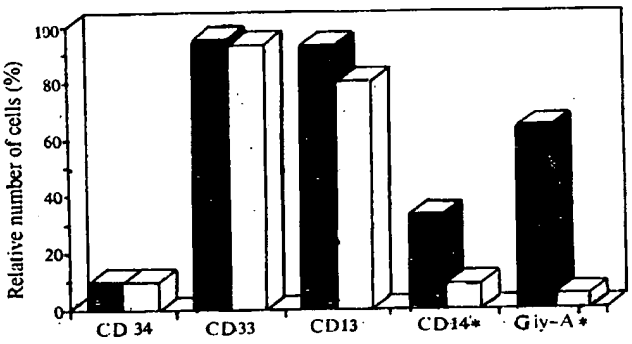

Fig. 2. Relative number of cells expressing 5 different myeloid and myeloid–associated cell surface markers after 11 days of incubation of CD34 cells ($^*P<0.01$). (■) IL–3+SCF+G–CSF, (□) IL–3+SCF

Serial changes in immunophenotype during culture. Cell maturation in culture was assessed by monitoring the serial expression of CD34, CD33, CD13, CD14 and Gly–A. The incidence of $CD34^+$ cells decreased from 97% to 54.5% during the first 5 days, and decreased rapidly to 1% to 2% by 11 days, and $CD34^+$ cells could not be detected afterwards. Expression of CD33 continued to increase to near 97% by day 11. Cells generated in culture progressively acquired CD13, CD14 and Gly–A antigen respectively. Gly–A antigen decreased rapidly after 15 days in suspension culture, probably owing to the fact that the liquid culture system was stroma–free and Epo–free and unable to induce erythroid progenitor to differentiate and proliferate further (Fig. 3).

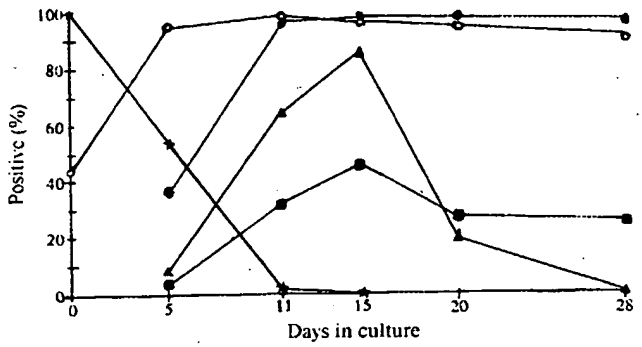

Fig. 3. The proportion changes of cells expressing CD34, CD33, CD13, CD14 and Gly–A during the culture, in the presence of IL–3, SCF and G–CSF. (*)CD34, (○)CD33, (●)CD13, (▲)Gly–A, (■)CD14

Number changes of BFU–E and CFU–MK. The results are shown in Fig. 4. BFU–E and CFU–GM generated 8.5– and 2.3–fold increase (in mean), respectively, in liquid culture for 11 days. Neither of them could be detected after that time.

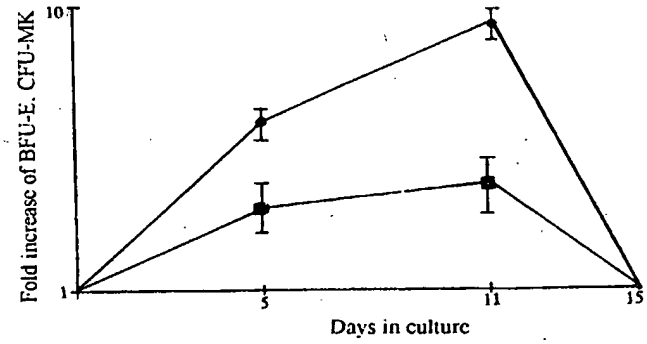

Fig. 4. The fold increase of BFU–E and CFU–MK In liquid culture of isolated $CD34^+$ cells, in the presence of IL–3, SCF and G–CSF. The results represent $\bar{x}\pm s$ of four seperate experiments performed in triplicate. ( ) BFU–E, (■) CFU–MK

DISCUSSION

The present study shows that G–CSF exhibits synergistic hematopoietic activity with other cytokines which act on more primitive progenitor

cells. In liquid–phase culture this combination enhances proliferation and differentiation along monocytic, granulocytic, erythrocytic and megakaryocytic lineages. On purified multipotent stem cells, SCF and IL–3 have a modest colony–stimulating effect when used alone,[2] and G–CSF has been shown to synergize with SCF and IL–3 in stimulating colonies derived from early progenitor cells enriched from G–CSF–mobilized PBSC, resulting in an increase in colony numbers (CSF–GM, BFU–E, CFU–MK) and nucleated cell numbers.

This study combines cell growth analysis with multimarker immunophenotyping changes, and represents an optional way to investigate the direct effect of cytokines on peripheral blood stem cell.

BFU–E and CFU–MK are usually generated for less than 2 weeks in the suspension culture. In most human long–term culture system, the duration of erythropoiesis and megakaryocytopoiesis is not sustained as long as that of granulopoiesis and monocytopoiesis.[3,4] The reasons are not very clear.

This stroma–free, cytokine–driven culture system can achieve a degree of amplification of CFU numbers in vitro before engraftment. Peripheral blood stem cell (PBSC) transplantation is suited to assess the efficacy of culture cells in vitro. The rapid postgraft hematopoietic recovery minimizes the period of cytopenia to be covered by culture cells to only 11 days. Such an approach may be applied to the amelioration of severe cytopenia after high–dose therapy.[5,6]

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(Received July 20, 1995)
本文编辑：杨振铎

Angiocardiographic Technique and Diagnosis of Pulmonary Atresia with Ventricular Septal Defect. Zhu Ming, Yang Zhenyong, Huang Lianxi. Department of Radiology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092. Chin J Radiol 1996; 30:102.

Purpose: To introduce a special angiocardiographic technique for diagnosis of pulmonary atresia with ventricular septal defect. **Materials and methods:** In 66 cases of pulmonary atresia with ventricular septal defect, aortogram, selective aortopulmonary collateral angiogram and pulmonary vein wedge angiogram were performed. **Results:** In the 66 cases, the pulmonary arterial trees were supplied by patent ducts arteriosus (24/66), aortopulmonary collaterals (26/66) and other small collaterals (12/66). Mixed supply type was in 4/66. The pulmonary arteries were confluent in 42 cases. **Conclusion:** Pulmonary vein wedge angiogram is a very effective and safe method for the diagnosis of some pulmonary atresia with ventricular septal defect.